oligonucleotides encoding mutated HCDR3. The pool of oligonucleotides encoding the HCDR3 library was mixed with the overlapping oligonucleotides encoding the combinatorial framework and other CDRs to generate a framework/HCDR3 library. The diversity of this library, termed Hu II, was 1.1×10^5 (Table I).

The CDR residues selected for mutagenesis of LCDR3 were Gln^{89}-Thr^{97} (Fig. 1, underlined). Oligonucleotides encoding LCDR3 were designed to mutate a single CDR residue in each clone as described above for HCDR3. Oligonucleotides encoding the LCDR3, HCDR3, and the combinatorial framework were used to create a framework/HCDR3/LCDR3 library, termed Hu III. The large number of framework/CDR3 combinations resulted in a library with a complexity of 3.1×10^7 (Table I).

Table I. Summary of phage-expressed anti-CD40 antibody libraries.

Library	Library Positions	Size*	Screened†
Hu I	framework	256	2.4×10^3
Hu II	framework, HCDR3	1.1×10^5	2.0×10^6
Hu III	framework, HCDR3, LCDR3	3.1×10^7	5.5×10^5

*Number of unique clones based on DNA sequence. Thirty-two codons are used to encode all 20 amino acids at each CDR position.

An additional library (Hu IV) was synthesized to further optimize the best variant (clone F4) identified from the Hu III library. Oligonucleotides encoding LCDR3, designed to mutate a single CDR residue

in each clone, were synthesized by introducing NN(G/T) at each position (Glaser et. al., (1992)) and were annealed to uridinylated F4 template (Kunkel, (1985)) which already contained a $^{96}R \rightarrow W$ mutation in LCDR3.

Combining mutations in LCDR3 and/or HCDR3 with the framework library increased the potential diversity of humanized anti-CD40 variants from 256 to greater than 10^7. In order to screen these larger libraries more efficiently a modified plaque lift assay, termed capture lift, was used (Watkins et. al., (1997)). Briefly, nitrocellulose filters (82-mm) were coated with goat anti-human kappa, blocked with 1% BSA, and were applied to an agar plate containing the phage-infected bacterial lawn. In the initial screen, phage were plated at 10^5 phage/100-mm plate. After the capture of phage-expressed anti-CD40 variant Fabs, the filters were incubated 3 h at 25°C with 5 ng/ml CD40-Ig in PBS containing 1% BSA. The filters were rinsed four times with PBS containing 0.1% Tween 20 and were incubated with goat anti-mouse IgG_{2b}-alkaline phosphatase conjugate (Southern Biotechnology) diluted 3000-fold in PBS containing 1% BSA for 1 h at 25°C. The filters were washed four times with PBS containing 0.1% Tween 20 and were developed as described (Watkins et. al., (1998)). To isolate individual clones, positive plaques from the initial screen were picked, replated at lower density ($<10^3$ phage/100-mm plate), and were screened by the same approach. Because the filters were probed with antigen at a concentration substantially below the *K*d of the Fab only variants displaying enhanced affinity were detectable. Multiple clones displaying higher affinities were identified following the screening of $>10^6$ variants from Hu II and $>10^5$ variants from the Hu III library using 82-mm filters containing 10^5 variants per filter (Table

I). Titration of the variants on immobilized CD40-Ig verified that multiple clones displayed affinities greater than the chimeric and humanized Fab (Fig. 2A, compare open squares, filled triangles with circles).

The framework/CDR mutations that conferred enhanced affinity were identified by DNA sequencing. Single-stranded DNA was isolated and the H and L chain variable region genes were sequenced by the fluorescent dideoxynucleotide termination method (Perkin-Elmer, Foster City, CA). Unique variable region sequences were identified in 10/13 Hu II variants and 4/5 Hu III variants. Both the Hu II and Hu III variants contained 1-5 murine framework residues and 0-2 CDR3 mutations. Representative examples are summarized in Table II. The affinities of bacterially-expressed chimeric Fab and certain variants from each of the libraries were characterized more thoroughly using surface plasmon resonance measurements to determine the association and dissociation rates of purified Fab with immobilized CD40-Ig as described above.

Chimeric anti-CD40 had a dissociation constant K_d = 48.3 nM and, consistent with the screening results, the variants all displayed higher affinities with K_d ranging from 0.24 nM to 10.5 nM (Table II). Further optimization of LCDR3 of Hu III clone F4 resulted in the identification of a higher affinity (K_d=0.1 nM) clone, L3.17, which contained a $^{94}F \rightarrow Y$ mutation. The improved affinities of the anti-CD40 variants were predominantly the result of slower dissociation rates. However, the association rates of most variants were also enhanced, increasing by as much as ≈3-fold (1.2 vs. 3.2 x 10^6 $M^{-1}s^{-1}$ for chimeric anti-CD40 and clone L3.17, respectively).